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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
A61K 39/385, 39/00, A61P 31/00 // C07K 16/00, 14/285, 14/575

A1

(11) International Publication Number: **WO 00/50077**

(43) International Publication Date: 31 August 2000 (31.08.00)

(21) International Application Number: PCT/EP00/01457

(22) International Filing Date: 22 February 2000 (22.02.00)

(30) Priority Data:

9904405.9	25 February 1999 (25.02.99)	GB
9904408.3	25 February 1999 (25.02.99)	GB
9904412.5	25 February 1999 (25.02.99)	GB
9919260.1	13 August 1999 (13.08.99)	GB

(71) Applicant *(for all designated States except US)*: SMITHKLINE BEECHAM BIOLOGICALS S.A. [BE/BE]; Rue de l'Institut 89, B–1330 Rixensart (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: COSTE, Michel [BE/FR]; SmithKline Beecham Biologicals S.A., Rue de l'Institut 89, B–1330 Rixensart (BE). LOBET, Yves [BE/BE]; SmithKline Beecham Biologicals S.A., Rue de l'Institut 89, B–1330 Rixensart (BE). VAN–MECHELEN, Marcelle, Paulette [BE/BE]; SmithKline Beecham Biologicals S.A., Rue de l'Institut 89, B–1330 Rixensart (BE). VERRIEST, Christophe [BE/BE]; SmithKline Beecham Biologicals S.A., Rue de l'Institut 89, B–1330 Rixensart (BE).

(74) Agent: DALTON, Marcus, Jonathan, William; SmithKline Beecham, Corporate Intellectual Property, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: IMMUNOGENS COMPRISING A PEPTIDE AND A CARRIER DERIVED FROM H.INFLUENZAE PROTEIN D

(57) Abstract

The present invention provides peptide immunogens linked to a carrier wherein the carrier is derived from Haemophilius Influenzae Protein D or fragments thereof.

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IMMUNOGENS COMPRISING A PEPTIDE AND A CARRIER DERIVED FROM H.INFLUENZAE PROTEIN D

The present invention relates to immunogens comprising a peptide and a carrier, in particular when the carrier is derived from Haemophilus Influenzae Protein D. The immunogens of the present invention may be used in pharmaceutical compositions for the prophylaxis or therapy of disease, especially in the form of vaccines. The invention further relates to their production, pharmaceutical compositions containing them, and their use in medicine.

Immunogens comprising short peptides are becoming increasingly common in the field of vaccine prophylaxis or therapy. The peptides which are commonly used may be the full length native immunogen, for example human peptidic hormones, or may be fragments of a larger antigen derived from a given pathogen, or from a large self-protein.

In many disease states it is often possible, and desirable, to design vaccines comprising a short peptide rather than the large protein. For example, immunoprophylaxis of allergy may beneficially comprise the use of short peptides of IgE (EP 0 477 231 B1), whereas the use of IgE itself as the immunogen may induce anaphylactic shock. In addition, a number of authors have described the use of the whole length Gonadotrophin hormone releasing hormone (GnRH, WO 95/20600), a short 10 amino acid long peptide, in the treatment of many cancers, or in immunocastration.

International Patent Application No. WO 99/27944 – (Athena Neurosciences) discloses that the N terminal 39 –43 amino acid fragement (Aβ) of the amyloid precursor protein may have utility in the prevention or treatment of Alzheimer's disease. Smaller fragments conjugated to a carrier are said to be useful. The application is based on the observation that PDAPP mice already possessing amyloid deposits injected with the N terminal 42 amino acid fragment of the amyloid precursor protein Aβ42 in various adjuvant formulations slows and prevents progressive amyloid deposition and consequential neuropathological changes in the aged PDAPP mouse brain. Additionally in young PDAPP injected with Aβ42 that no or extremely little amyloid was deposited in their brains and that the pathological

consequences were absent. Thus the data in the application seem to indicate that Aβ has both a prophylactic and therapeutic potential.

Amongst the problems associated with the peptide approach to vaccination, is the fact that peptides *per se* are poor immunogens. Generally the sequence of the peptides are chosen such that they include a B-cell epitope to provide a target for the generation of anti-peptide antibody responses, but because of their limited size rarely encompass sufficient T-cell epitopes in order to provide the necessary cytokine help in the induction of strong B-cell responses. Strategies which have been designed to overcome this lack of immunogenicity include the linking of the peptide to large highly immunogenic protein carriers, which provide bystander T-cell help, and/or the use of strong adjuvants in the vaccine formulation.

Examples of these highly immunogenic carriers which are currently commonly used for the production of peptide immunogens include the Diptheria and Tetanus toxoids (DT and TT respectively), Keyhole Limpet Haemocyanin (KLH), and the purified protein derivative of Tuberculin (PPD). A number of problems are associated with each of these commonly used carriers, including both problems in production of GMP constructs and also in immunological characteristics of the constructs. The present invention provides a new carrier for use in the preparation of peptide-based immunogen constructs, that does not suffer from the disadvantages associated with such large highly immunogenic carriers.

Amongst the disadvantages of these carriers is the fact that antigen specific immune responses may be suppressed by the presence of pre-existing antibodies directed against the carrier. For example immune against Tetanus toxin (TT) (Di John *et al*; Lancet, December 16, 1989) suppress the immune response to a peptide conjugated to TT. This is a problem which is made evident by the fact that in the population at large, a very high percentage of people will have pre-existing immunity to both DT and TT as people are routinely vaccinated with these antigens. In the UK for example 95% of children receive the DTP vaccine comprising both DT and TT. Other authors have described the problem of epitope suppression to peptide vaccines in animal models (Sad *et al*, Immunology, 1991; 74:223-227; Schutze *et al*, J. Immunol. 135: 4, 1985; 2319-2322).

In addition, for vaccines that require regular boosting, the use of highly immunogenic carriers such as TT and DT are likely to suppress the peptide antibody response after several injections. These multiple vaccinations may also be accompanied by undesireable reactions such as delayed type hyperresponsiveness (DTH).

KLH is known as potent immunogen and has already been used as a carrier for peptides derived from IgE (EP 0477231B1) in human clinical trials. However, some adverse reactions (DTH-like reactions or IgE sensitisation) as well as antibody responses against KLH which could compete with the anti-decapeptide antibody have been observed.

The selection of a carrier protein, therefore, for a peptide based vaccine will require a balance between the necessity to use a carrier working in all patients (broad MHC recognition) and the induction of high levels of anti-peptide antibody responses and of low antibody response against the carrier.

The carriers used previously for peptide based vaccines, whilst they may induce high levels of antipeptide antibody responses on primary immunisation, therefore have many disadvantages.

The present invention provides a protein D from Haemophilus Influenzae, or fragments thereof, as a carrier for peptide based vaccines which induces high anti-peptide immune responses with a moderate or low anti-carrier response. Preferably the peptide is a non-Haemophilius Influenzae derived peptide.

One of the fields of research where a great deal of effort has been made in designing peptide based vaccines in the prophylaxis and therapy of allergic responses. In a response to allergens, the symptoms of an allergic response are brought about by the release of mediators (such as histamine) from immune cells into the surrounding tissues and vascular structures. Histamine is normally stored in mast cells or basophils, until such time as the release is triggered by interaction with allergen specific IgE.

The role of IgE in the mediation of allergic responses, such as asthma, food allergies, type-I hypersensitivity and sinus inflammation, is well known. On encountering an antigen, such as pollen or dust mite allergens, B cells commence the synthesis of allergen specific IgE. The allergen specific IgE then binds to its FcεRI

receptor (the high affinity receptor) on basophils and mast cells. Any subsequent encounter with allergen leads to the triggering of histamine release from the mast cells or basophils, and cross-linking of neighbouring IgE/ FcεRI complexes (EP 0 477 231 B1).

A number of passive or active immunotherapeutic and immunoprophylactic approaches which interfere with this IgE-mediated histamine release mechanism have been investigated. These approaches include the use of specific IgE peptides for active immunisation, in order to stimulate auto anti-IgE antibodies which are capable of inhibiting histamine release by interfering with the IgE-mediated trigger signal.

EP 0 477 231 B1 describes one such approach. Peptides derived from the Cε4 domain of IgE (residues 497-506, also known as the Stanworth decapeptide), conjugated to Keyhole Limpet Haemocyanin (KLH). A number of carriers for the IgE peptides are suggested including KLH, Tetanus toxoid, Diptheria toxoid, albumins, haemocyanins such as Keyhole Limpet Haemocyanin (KLH), polymers of amino acids, and preferably purified protein derivative of tuberculin (PPD).

WO 95/26365 further continues investigation of the Cε4 (497-506) peptide and describes immunogens which is rendered substantially free of carrier protein by the addition of universal T-helper epitopes. The immunogens of WO 95/26365 are in the general formula:

$(A)_n$-$(Th)_m$-$(B)_o$-$(Stan)_p$

where A is an amino acid; Th is a T-helper epitope; B is an amino acid; and Stan is the Stanworth decapeptide. n=1 to 10; m=1 to 4; o=0 to 10; and p=1 to 3.

WO 98/24808 describes oligopeptides derived from Cε3, which interact with the high or low affinity receptors of IgE. These oligopeptides are expressed as fusion proteins together with the expression partner Glutathione-S-transferase (GST). WO 97/31948 describes IgE peptide immunogens conjugated to a protein carrier, the carriers described are TT, DT, KLH and PPD.

Another important area for peptide vaccine research has been in the field of human peptide hormone vaccines. For example the peptide hormone, Gonadotrophin hormone releasing hormone (GnRH) has been used for the immunotherapy of Gonadal Steroid Hormone dependent diseases, such as many cancers (including prostate cancer) and immunocontraception. WO 95/20600 describes the use of GnRH

conjugated to a carrier protein in order to render it immunogenic. The example of a carrier used in the examples is Diptheria toxoid (DT).

Other work in the art use similar carriers including PPD, TT, DT and the DT derivative, CRM197, bovine serum albumin (BSA), Equine Serum Albumin (ESA), Equine gamma globulin, Ovalbumin (OVA) Keyhole Limpet Haemocyanin (KLH), porcine thyroglobuin, bacterial adhesins, viral haemagglutinin, hepatitis B surface antigen, bacterial toxin such as LTB, outer membrane lipoprotein of *E.Coli*, TraT protein, leukotoxin polypeptide, Pseudomonas exotoxin protein PE38QQR (EP 0 293 530 A2; EP 0 181 236 B1; WO 90/03182; EP 0 222 835 B1; EP 0 446 313 B1; WO 96/24675; US 5,324,512; US 4,975,420; WO 97/15316 WO 97/15325; WO 97/15317; WO 90/02187) .

In the context of utilising Aβ peptides, International patent application No: WO99/27944 contemplates conjugating the peptide to a conventional carrier protein. These carrier proteins mentioned in this application suffer from the aforementioned disadvantages.

The present invention overcomes these problems associated with peptide carriers of the prior art. The problems are surprisingly overcome by the use of Protein D as a peptide carrier for the preparation of immunogens.

Protein D is an IgD–binding protein from *Haemophilus influenzae* and has been patented by Forsgren WO 91/18926 (EP 0 594 610 B1). The patent describes the cloning, expression, and protein sequence of Protein D. It suggests its uses could be methods of detecting IgD using its selective affinity for IgD (as for Protein A and Protein G) and in the stimulation of the immune system through interaction with B-lymphocytes.

EP 0 594 610 B1 further describes the Protein D gene, and suggests that it may be fused to other genes and expressed to form fusion proteins.

Akkoyunlu et al. (Infection and Immunity, 1997, 65, 12, 5010-5016) and Akkoyunlu et al. (Infection and Immunity, 1996, 64, 11 4586-4592) describe the large scale purification of the non-acylated form of Protein D, and its use in vaccines. Immunised mice and rats had high levels of bactericidal protein D specific IgA and IgG.

The present invention provides the use of protein D as a carrier for peptide immunogens. Said immunogens have the advantage of inducing high levels of anti-peptide immune responses whilst inducing a moderate humoral response against itself. In the context of an allergy vaccine, protein D as a carrier has the additional advantage of inducing low anti-carrier IgE responses in comparison to known carriers, such as KLH.

In some circumstances, for example, in recombinant expression systems it may be desirable to use fragments of protein D, for example protein D 1/3rd (comprising the N-terminus 100-110 amino acids of protein D (GB 9717953.5)). Accordingly there is provided by the present invention protein D or fragment thereof and a peptide immunogen for use in the manufacture of an immunogen.

The immunogens of the present invention, therefore, comprise a peptide conjugated to protein D as a carrier. The peptides are preferably chemically conjugated.

The term "peptide" within the meaning of the present invention is a polymer of amino acids containing a sequence of amino acids less than 100 amino acids in length preferably between 2-50 amino acids in length, and more preferably between 2-45 amino acids in length more preferably 2-30, and most preferably between 5-25 amino acids in length. The peptides of the present invention may be either lipidated or non-lipidated. It will be appreciated however that peptide residues may be joined in any convenient chemical way, for example via an ester linkage and the like, but typically will be joined by a peptide bond.

The peptides may be full-length sequences of naturally occurring peptides like peptide hormones. Alternatively, the peptides may be derived from naturally occurring self proteins or proteins derived from pathogens. In certain context it may be desirable to use non-natural amino acids such as D-amino acids.

In the context of an Aβ peptide the peptide also preferably include the naturally occurring peptide of between 39 and 43 amino acids in length. Thus the preferred sequences correspond to the naturally occurring forms i.e. those sequence corresponding to amino acids 1 to 39, 1 to 40, 1 to 41 1 to 42, 1 to 43 of the amyloid precursor protein and which are disclosed in Hardy et al ., TINS 20, 155 – 158, 1997.

Immunogenic fragments of Aβ43 can also be coupled to Protein D in accordance with the invention. Preferred fragments include peptides incorporating residues selected from the group: Aβ 1-5; 1-12, 13 –28; 17 –28 and 33-42. Such constructs find utility in the treatment or prevention of Alzheimer's disease. Peptide mimotopes of Aβ43 or fragments thereof are also contemplated.

Particularly preferred examples of peptides for use as immunogens of the present invention include GnRH or mimotopes or analogues thereof: GnRH has the sequence EHWSYGLRPG (SEQ ID NO. 1). Alternatively, tandem repeats, of GnRH such as E-H-W-S-Y-G-L-R-P-G-*S*-***C***-*S*-E-H-W-S-Y-G-L-R-P-G-NH2 (SEQ ID NO. 2) may be conjugated to a protein D molecule, typically through a central cysteine. Tandem dimers are also contemplated by the present invention such as E-H-W-S-Y-G-L-R-P-G-Q-H-W-S-Y-G-L-R-P-G-*S*-***C***-E-H-W-S-Y-G-L-R-P-G-Q-H-W-S-Y-G-L-R-P-G-NH2 (SEQ ID NO. 3). These advantageously are conjugated to Protein D via the central Cysteine. Such constructs find utility, inter alia, in the treatment of Prostate cancers. Alternatively, GnRH may be linked to Protein D via a peptide spacer. Typical spacers includes $Cys(Pro)_4$ Ser Ser.

In an alternative preferred embodiment, the peptides are derived from mammalian IgE, such that the immunogens are capable of stimulating a non-anaphylactic anti-IgE antibody response in a vaccinated individual. For example, the stanworth decapeptides as described in EP 0 477 231 B1, for example KTKGSGFFVF (SEQ ID NO. 4) or mimotopes thereof. Other IgE peptides are described in WO 97/31948 and WO 96/14333, and are useful in immunogens of the present invention.

Preferred epitopes from IgE are for example, those which are surfaced exposed and have the following sequences:

Peptide	***Sequence***	***Length exposed***	***IgE Domain***
P1	EDGQVMDVD (SEQ ID NO. 5)	9	C2
P2	STTQEGEL (SEQ ID NO. 6)	8	C2
P3	SQKHWLSDRT (SEQ ID NO. 7)	10	C2
P4	GHTFEDSTKKCADSNPRGV (SEQ ID NO. 8)	19	C2/C3

P1 and P2 are located within the C2 domain of IgE, within a region which has not previously been reported as being useful for the active vaccination based immunoprophylaxis of allergy.

The present invention, therefore, includes the native peptides themselves, and any mimotope thereof. The meaning of mimotope is defined as a peptide sequence which is sufficiently similar to the native peptide (sequentially or structurally), which is capable of being recognised by antibodies which recognise the native peptide; or are capable of raising antibodies, when coupled to a suitable carrier, which antibodies are capable of recognising the native peptide.

Peptide mimotopes may be designed for a particular purpose by addition, deletion or substitution of elected amino acids. Thus, the peptides may be modified for the purposes of ease of conjugation to a protein carrier. For example, it may be desirable for some chemical conjugation methods to include a terminal cysteine. In addition it may be desirable for peptides conjugated to a protein carrier to include a hydrophobic terminus distal from the conjugated terminus of the peptide, such that the free unconjugated end of the peptide remains associated with the surface of the carrier protein. Thereby presenting the peptide in a conformation which most closely resembles that of the peptide as found in the context of the whole native molecule. Examples of such modified peptides include:

Examples of such modified peptides useful as anti allergy medicament include:

Peptide	*Sequence*	*Description*
P10	CADSNPRGV (SEQ ID NO. 9)	P4 variant
P15	CLEDGQVMDVDLL-NH2 (SEQ ID NO. 10)	P1 variant
P16	CSTTQEGELA- NH2 (SEQ ID NO. 11)	P2 variant
p17	CSQKHWLSDRT- NH2 (SEQ ID NO. 12)	P3 variant

The immunogens of the present invention may alternatively contain a peptide capable of eliciting an immune response against a human pathogen. Such peptides may be derived from the following group: an HIV antigen (such as tat, nef, gp120 or gp160), a human herpes virus antigen (such as gD or derivatives thereof or Immediate Early protein such as ICP27 from HSV1 or HSV2), a cytomegalovirus antigen (such as gB or derivatives thereof), a Rotavirus antigen, an Epstein Barr virus antigen (such

as gp350), Varicella Zoster Virus antigens (such as gpI, II and IE63), or from a hepatitis virus such as hepatitis B virus (for example Hepatitis B Surface antigen), hepatitis A virus antigen, hepatitis C virus and hepatitis E virus, or from other viral pathogens, such as paramyxoviruses: Respiratory Syncytial virus (such as F and G proteins), parainfluenza virus, measles virus, mumps virus, human papilloma viruses (for example HPV6, 11, 16, 18), flaviviruses (e.g. Yellow Fever Virus, Dengue Virus, Tick-borne encephalitis virus, Japanese Encephalitis Virus) or Influenza virus antigens, or peptides derived from bacterial pathogens such as *Neisseria spp,* including *N. gonorrhea* and *N. meningitidis* (for transferrin-binding proteins, lactoferrin binding proteins, PilC, adhesins); *Streptococcus spp,* including *S. pneumoniae* (streptolysin or choline-binding proteins), *S. pyogenes* (for example M proteins, C5A protease), *S. agalactiae, S. mutans; Haemophilus spp, including H. influenzae type B, non typeable H. influenzae* (for example OMP26, high molecular weight adhesins, P5, P6), *H. ducreyi; Moraxella spp, including M catarrhalis, also known as Branhamella* catarrhalis (for example high and low molecular weight adhesins and invasins*); Bordetella spp, including B. pertussis* (for example pertactin, pertussis toxin, filamenteous hemagglutinin, adenylate cyclase, fimbriae), *B. parapertussis and B. bronchiseptica; Mycobacterium spp., including M. tuberculosis* (for example ESAT6, Antigen 85A, -B or -C), *M. bovis, M. leprae, M. avium, M. paratuberculosis, M. smegmatis; Legionella spp,* including *L. pneumophila; Escherichia spp,* including *enterotoxic E. coli* (for example colonization factors, heat-labile toxin or derivatives thereof, heat-stable toxin or derivatives thereof), enterohemorragic *E. coli,* enteropathogenic *E. coli* (for example shiga toxin-like toxin or derivatives thereof); *Vibrio spp,* including *V. cholera* (for example cholera toxin or derivatives thereof*); Shigella spp,* including *S. sonnei, S. dysenteriae, S. flexnerii; Yersinia spp,* including *Y.* enterocolitica (for example a Yop protein) , *Y. pestis, Y. pseudotuberculosis; Campylobacter spp,* including *C. jejuni* (for example toxins, adhesins and invasins) and *C. coli; Salmonella spp,* including *S. typhi, S. paratyphi, S. choleraesuis, S. enteritidis; Listeria spp.,* including *L. monocytogenes; Helicobacter spp,* including *H.* pylori (for example urease, catalase, vacuolating toxin); *Pseudomonas spp,* including *P. aeruginosa; Staphylococcus spp.,* including *S. aureus, S. epidermidis; Enterococcus spp.,* including *E. faecalis, E. faecium; Clostridium*

spp., including *C.* tetani (for example tetanus toxin and derivative thereof), *C. botulinum* (for example botulinum toxin and derivative thereof), *C. difficile* (for example clostridium toxins A or B and derivatives thereof); *Bacillus spp.*, including *B. anthracis* (for example botulinum toxin and derivatives thereof); *Corynebacterium spp.*, including *C. diphtheriae* (for example diphtheria toxin and derivatives thereof); *Borrelia spp.*, including *B. burgdorferi* (for example OspA, OspC, DbpA, DbpB), *B. garinii* (for example OspA, OspC, DbpA, DbpB), *B. afzelii* (for example OspA, OspC, DbpA, DbpB), *B. andersonii* (for example OspA, OspC, DbpA, DbpB), *B. hermsii; Ehrlichia spp.*, including *E. equi* and the agent of the Human Granulocytic Ehrlichiosis; *Rickettsia spp*, including *R. rickettsii; Chlamydia spp., including C. trachomatis* (for example MOMP, heparin-binding proteins), *C. pneumoniae* (for example MOMP, heparin-binding proteins), *C. psittaci; Leptospira spp.*, including *L. interrogans; Treponema spp.*, including *T. pallidum* (for example the rare outer membrane proteins), *T. denticola, T. hyodysenteriae;* or derived from parasites such as *Plasmodium spp.*, including *P. falciparum; Toxoplasma spp.*, including *T. gondii (for example SAG2, SAG3, Tg34); Entamoeba spp.*, including *E. histolytica; Babesia spp.*, including *B. microti; Trypanosoma spp.*, including *T. cruzi; Giardia spp.*, including *G. lamblia; Leshmania spp.*, including *L. major; Pneumocystis spp.*, including *P. carinii; Trichomonas spp.*, including *T. vaginalis; Schisostoma spp.*, including *S. mansoni,* or derived from yeast such as *Candida spp.*, including *C. albicans; Cryptococcus spp.*, including *C. neoformans.*

Vaccines of the present invention further comprise peptides derived from parasites that cause Malaria. Peptides may be derived from the circumsporozoite (CS) protein of *P.falciparum,* or TRAP antigens (WO 90/01496). Other plasmodia antigens that are likely candidates to be donors of peptides for a multistage Malaria vaccine are *P. faciparum* MSP1, AMA1, MSP3, EBA, GLURP, RAP1, RAP2, Sequestrin, PfEMP1, Pf332, LSA1, LSA3, STARP, SALSA, PfEXP1, Pfs25, Pfs28, PFS27/25, Pfs16, Pfs48/45, Pfs230 and their analogues in Plasmodium spp.

The immunogens of the present invention may also contain an anti-tumour peptide and be useful for the immunotherapeutic treatment cancers. For example, the adjuvant formulation finds utility with tumour rejection antigens such as those for prostrate, breast, colorectal, lung, pancreatic, renal or melanoma cancers. Exemplary

antigens include MAGE 1 and MAGE 3 or other MAGE antigens for the treatment of melanoma or MAGE expressing tumour (particularly the peptide EVDPIGHLY (Seq ID No 13) (US 5,662,907), PRAME, BAGE or GAGE (Robbins and Kawakami, 1996, Current Opinions in Immunology 8, pps 628-636; Van den Eynde et al., International Journal of Clinical & Laboratory Research (submitted 1997); Correale et al. (1997), Journal of the National Cancer Institute 89, p293. Indeed these antigens are expressed in a wide range of tumour types such as melanoma, lung carcinoma, sarcoma and bladder carcinoma. Other peptides derived from Tumor-Specific antigens are suitable for use with adjuvant of the present invention and include, but are not restricted to Prostate specific antigen (PSA) or Her-2/neu, KSA (GA377), MUC-1 and carcinoembryonic antigen (CEA). Accordingly in one aspect of the present invention there is provided an immunogen comprising a peptide derived from a tumour rejection antigen coupled to protein D.

Peptides used in the present invention can be readily synthesised by solid phase procedures well known in the art. Suitable syntheses may be performed by utilising "T-boc" or "F-moc" procedures. Cyclic peptides can be synthesised by the solid phase procedure employing the well-known "F-moc" procedure and polyamide resin in the fully automated apparatus. Alternatively, those skilled in the art will know the necessary laboratory procedures to perform the process manually. Techniques and procedures for solid phase synthesis are described in 'Solid Phase Peptide Synthesis: A Practical Approach' by E. Atherton and R.C. Sheppard, published by IRL at Oxford University Press (1989). Alternatively, the peptides may be produced by recombinant methods, including expressing nucleic acid molecules encoding the mimotopes in a bacterial or mammalian cell line, followed by purification of the expressed mimotope. Techniques for recombinant expression of peptides and proteins are known in the art, and are described in Maniatis, T., Fritsch, E.F. and Sambrook et al., *Molecular cloning, a laboratory manual*, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

The ratio of peptide to protein D carrier is typically in the order of between 1:1 to 1:20, but preferably between 2 to 10 peptides per Protein D carrier, typically 3 to 8. Different peptides may be coupled to the same carrier molecule to provide a hybrid immunogen.

The covalent coupling of the peptide to Protein D or fragment thereof can be carried out in a manner well known in the art. Thus, for example, for direct covalent coupling it is possible to utilise a carbodiimide, glutaraldehyde or (N-[γ-maleimidobutyryloxy] succinimide ester, utilising common commercially available heterobifunctional linkers such as CDAP and SPDP (using manufacturers instructions). After the coupling reaction, the immunogen can easily be isolated and purified by means of a dialysis method, a gel filtration method, a fractionation method etc.

In a preferred embodiment the vaccines of the present invention are preferably used for the prophylaxis or therapy of allergy. Such vaccines may comprise allergen specific peptide (for example from Der p1) or allergen non-specific peptides (for example the stanworth decapeptide or P1 to P4 or mimotope thereof).

Vaccines of the present invention comprise an immunogen as described above and an adjuvant. Suitable adjuvants are well known in the art (Vaccine Design – The Subunit and Adjuvant Approach, 1995, Pharmaceutical Biotechnology, Volume 6, Eds. Powell, M.F., and Newman, M.J., Plenum Press, New York and London, ISBN 0-306-44867-X).

Preferred adjuvants for use with immunogens of the present invention include: aluminium or calcium salts (eg hydroxide or phosphate), oil in water emulsions (WO 95/17210, EP 0 399 843), or particulate carriers such as liposomes (WO 96/33739). Immunologically active saponin fractions (*e.g.* Quil A) having adjuvant activity derived from the bark of the South American tree Quillaja Saponaria Molina are particularly preferred. Derivatives of Quil A, for example QS21 (an HPLC purified fraction derivative of Quil A), and the method of its production is disclosed in US Patent No.5,057,540. Amongst QS21 (known as QA21) other fractions such as QA17 are also disclosed. 3 De-O-acylated monophosphoryl lipid A is a well known adjuvant manufactured by Ribi Immunochem, Montana. It can be prepared by the methods taught in GB 2122204B. A preferred form of 3 De-O-acylated monophosphoryl lipid A is in the form of an emulsion having a small particle size less than 0.2μm in diameter (EP 0 689 454 B1).

Adjuvants also include, but are not limited to, muramyl dipeptide and saponins such as Quil A, bacterial lipopolysaccharides such as 3D-MPL (3-O-deacylated

monophosphoryl lipid A), or TDM. As a further exemplary alternative, the protein can be encapsulated within microparticles such as liposomes, or in non-particulate suspensions of polyoxyethylene ether (UK Patent Application No. 9807805.8). Particularly preferred adjuvants are combinations of 3D-MPL and QS21 (EP 0 671 948 B1), oil in water emulsions comprising 3D-MPL and QS21 (WO 95/17210, PCT/EP98/05714), 3D-MPL formulated with other carriers (EP 0 689 454 B1), or QS21 formulated in cholesterol containing liposomes (WO 96/33739), or immunostimulatory oligonucleotides (WO 96/02555).

Examples of suitable pharmaceutically acceptable excipients include water, phosphate buffered saline, isotonic buffer solutions. The vaccines of the present invention may also be administered via the oral route. In such cases the pharmaceutically acceptible excipient may also include alkaline buffers, or digestible capsules.

The formulations of the present invention maybe used for both prophylactic and therapeutic purposes. Accordingly, the present invention provides for a method of treating a mammal susceptible to or suffering from an infectious disease or chronic disorders such as cancer, allergy, or autoimmune disease. In particular compositions (depending on the peptide used) of the present invention are useful in the treatment and prevention of Alzheimer's, Allergy and Prostate Carcinoma. In a further aspect of the present invention there is provided a vaccine as herein described for use in medicine. Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978.

In a further aspect of the present invention there is provided an immunogen or vaccine as herein described for use in medicine.

The vaccine preparation of the present invention may be used to protect or treat a mammal susceptible to, or suffering from allergies, by means of administering said vaccine via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts.

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in

typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-1000 μg of protein, preferably 1-500 μg, preferably 1-100 μg, of which 1 to 50μg is the most preferable range. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Conjugation of proteins to macromolecules is disclosed by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

Example 1, *Preparation of peptide/carrier immunogens and vaccine formulations*

1.1 Preparation of Protein D for use as a peptide carrier

Protein D may be produced and purified using methods described in EP 0 594 610 B1.

1.2 Preparation of IgE Peptides for conjugation to the carrier

The peptides which have been used in these examples are peptides derived from human or rodent IgE Cε4. These immunogens have previously been shown to be capable of stimulating antibody responsès which are capable of inhibiting histamine release from IgE bearing mast cells (EP 0 477 231 B1).

Peptides were chemically synthesised by NeoSystem Laboratoire (Strasbourg, France). The immunogen peptide have the sequences represented in the table 1.

Table 1, *Peptide sequences*

	Sequence	***Source***	***Molecular Weight***
PEP1 (deca)	KTKGSGFFVF-NH2 (SEQ ID NO. 14)	Human IgE	1117.3
PEP2 (deca)	CKTKGSGFFVF-NH2 (SEQ ID NO. 15)	Human IgE	1219.5
PEP3 (dodeca)	CKSNGSNQGFFIF-NH2 (SEQ ID NO. 16)	Rodent IgE	1447.6
PEP4 (dodeca	KSNGSNQGFFIF-NH2 (SEQ ID NO. 17)	Rodent IgE	1345.4

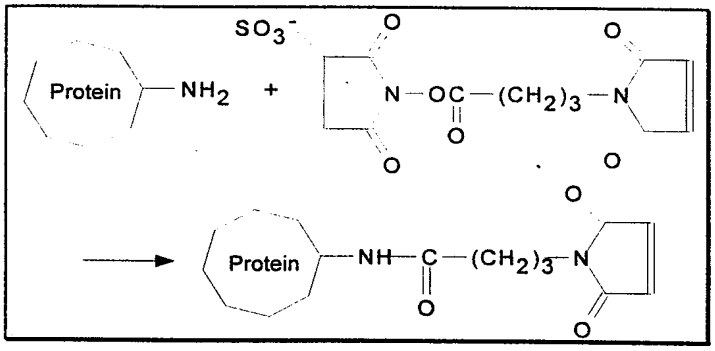
The peptides were stored dry and frozen at -20°C until used in the conjugation procedure.

1.3 Synthesis of conjugates using a succinimide-maleimide cross-linker

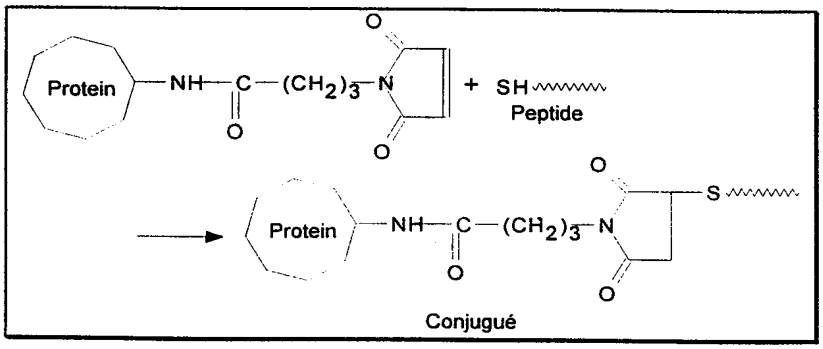
Protein D may be conjugated directly to peptides to form antigens of the present invention by using a maleimide-succinimide cross-linker. This chemistry allows controlled NH_2 activation of carrier residues by fixing a succinimide group. Maleimide groups is a cystein-binding site. Therefore, for the purpose of the following examples, the IgE peptides to be conjugated required the addition of an N-terminal cystein (PEP2 and PEP3).

The coupling reagent is a selective heterobifunctional cross-linker, one end of the compound activating amino group of the protein carrier by an succinimidyl ester and the other end coupling sulhydryl group of the peptide by a maleimido group. The reactional scheme is as the following :

a. Activation of the protein by reaction between lysine and succinimidyl ester :



b. Coupling between activated protein and the peptide cystein by reaction with the maleimido group :



1.4 Preparation of peptide PEP2-Protein D conjugate "low ratio"

The protein D is dissolved in a phosphate buffer saline at a pH 7.2 at a concentration of 2.5 mg/ml. The coupling reagent (N-[γ-maleimidobutyryloxy] succinimide ester - GMBS) is dissolved at 102.5 mg/ml in DMSO and added to the protein solution. 1.025 mg of GMBS is used for 1 mg of Protein D. The reactional solution is incubated 1 hour at room temperature. The by-products are removed by a desalting step onto a sephacryl 200HR permeation gel. The eluant used is a phosphate buffer saline Tween 80 0.1 % pH 6.8. The activated protein is collected and pooled. The peptide is dissolved at 4 mg/ml in 0.1 M acetic acid to avoid di-sulfure bond formation. A molar ratio of between 2 to 4 peptides per 1 activated Protein D is used for the coupling.

The peptide solution is slowly added to the protein and the mixture is incubated 1 h at 25°C. The pH is kept at a value of 6.6 during the coupling phase. A quenching step is performed by addition of cystein (0.1 mg cystein per mg of activated PD dissolved at 4 mg/ml in acetic acid 0.1 M), 30 minutes at 25°C and a pH of 6.5. Two dialysis against NaCl 150 mM Tween 80 0.1 % are performed to remove the excess of cystein or peptide.

The last step is a sterile filtration on a 0.22 µm membrane. The final product is a clear filtrable solution conserved at 4°C. The final ratio of peptide/PD in the construct is determined by Amino acid analysis.

1.5 Preparation of peptide PEP2-Protein D conjugate "high ratio"

The activated PD is prepared as in example 1.4. The activated Protein D is passed through a 0.22 µm membrane. Peptide is also diluted in acetic acid 0.1 M and filtrated on a 0.22 µm membrane. The two solutions are then slowly mixed to obtain a final molar peptide/Protein D ratio of 8. The pH is adjusted to 6.5. After 1 h at 25°C, the solution is conserved at 4°C.

The figure 1, shows the evolution of protein concentration according to increasing initial peptide / Protein D ratio. At a ratio of 4 peptide per 1 PD, the majority of the conjugate is soluble and is, therefore, found in the supernatant. At a ratio 8/1, nearly all of protein content is precipitated. At a higher ratio, the conjugate is precipitated and the excess of unconjugated peptide is found in the supernatant.

1.6 Production of Oil in Water (o/w) emulsion adjuvants

The oil in water emulsion adjuvant formulations used in the subsequent examples were each made comprising the following oil in water emulsion component: 5% Squalene, 5% α-tocopherol, 2.0% polyoxyethylene sorbitan monooleate (TWEEN 80).

The emulsion was prepared as follows as a 2 fold concentrate. All examples used in the immunological experiments are diluted with the addition of extra components and

diluents to give either a 1x concentration (equating to a squalene:QS21 ratio (w/w) of 240:1) or further dilutions thereof.

Briefly, the TWEEN 80 is dissolved in phosphate buffered saline (PBS) to give a 2% solution in the PBS. To provide 100 ml of a two fold concentrate emulsion, 5ml of DL alpha tocopherol and 5ml of squalene are vortexed to mix thoroughly. 95ml of PBS/TWEEN solution is added to the oil and mixed thoroughly. The resulting emulsion is then passed through a syringe needle and finally microfluidised by using an M110S Microfluidics machine. The resulting oil droplets have a size of approximately 145-180 nm (expressed as z av. measured by PCS) and is termed SB62.

The other adjuvant/vaccine components (QS21, 3D-MPL or antigen) are added to the emulsion in simple admixture.

1.7 Alum adjuvant may be bought from Superfos.

1.8 *ELISA assay to determine anti-antigen antibody responses*

The anti-peptide and anti-carrier immune responses were investigated using an ELISA technique outlined below.

- Microtiterplates (Nunc) are coated with the specific antigen in PBS (4° overnight) with either:

Streptavidin at 2μg/ml (followed by incubation with biotinylated peptide (1μM) for 1 hour at 37°C), or

KLH, or

Protein D.

- Wash 3X PBS-Tween 20 0.1%.
- Saturate plates with PBS-BSA 1%-Tween 20 0.1% (Sat buffer) for 1 hr at 37°.
- Add 1° antibody = sera in two-step dilution (in Sat buffer), incubate 1 hr 30 minutes at 37°.
- Wash 3X.

- Add 2° anti-mouse Ig (or anti-mouse isotype specific monoclonal antibody) coupled to HRP. Incubate 1 hr at 37°.
- Wash 5X.
- Reveal with TMB (BioRad) for 10 minutes at room temperature in the dark.
- Block reaction with 0.4N H_2SO_4.

A monoclonal anti-human decapeptide antibody (Dec7B) was used as reference in deca/dodeca ELISA. This makes it possible to calculate anti-decapeptide antibody responses either in µg specific antibody per 1ml or serum (µg/ml), or as a midpoint titre. Anit-Protein D and anti-KLH responses are calculated as midpoint titers.

1.9 Production of peptide-KLH conjugates

Peptide-KLH conjugates were made according to manufacturer's instructions.

Example 2, Immunogenicity studies with Stanworth decapeptide conjugates adsorbed onto alum.

Three groups of 10 Balb/C mice were vaccinated with the PEP2 human decapeptide – protein D conjugates produced according to the methods described in example 1. The vaccines comprised 25µg of antigen in 100µl volumes, and were given s.c on three occasions on days 0, 21, and 42 (V1, V2, and V3).

Table 2, *Vaccine compositions*

Name of group	***Number of peptide:Protein D***	***Adjuvant***
ProtD-Deca 007	2	$Al(OH)_3$ (100 µg)
ProtD-Deca 008	8	$Al(OH)_3$ (100 µg)
KLH-Deca	5	$Al(OH)_3$ (100 µg)

Bleedings were taken 21, 35, and 49 days post commencement of immunisation (21 post V1, 14 post V2, and 7 post V3).

The results for generation of antibody responses are shown in Table 3 for individual responses at time points 21 post V1, 14 post V2, and 7 post V3. Figure 2 (anti-

decapeptide IgG responses and anti-carrier IgG responses) shows the comparison of the antibody responses induced by the three vaccines in pooled sera.

The isotype of the antibodies produced was investigated in the ELISA system using anti-mouse isotype monoclonal antibodies (see table 3). These studies were performed on pooled serum samples.

Table 3, Anti-decapeptide responses generated by antigen conjugates adsorbed onto alum (7 post V3)

	Anti-Decapeptide (μg/ml)				*Isotype %*		
	IgG1	*IgG2a*	*IgG2b*	*total*	*IgG1*	*IgG2a*	*IgG2b*
ProtD-Deca 007	59	0	0	59	100	0	0
ProtD-Deca 008	151	0	0	151	100	0	0
KLH-Deca	10	0	0	10	100	0	0

The ratio's of Mid-point titres of anti-peptide:anti-carrier IgG responses were measured (results see table 4). This ratio is a measure of the relative levels of anti-peptide or anti-carrier immune response which were generated by vaccination. A high ratio (>1) indicates that strong anti-peptide response is being generated with respect to that induced against the carrier.

Table 4, Midpoint titers and ratio anti-deca versus anti-carrier response

Time point	**Vaccine formulation**	**Titre Mid point**		**ratio**
		IgG anti Deca	IgG anti carrier	IgG anti Deca : IgG anti Carrier
21 Post 1	KLH-Deca / Alum	5	1155	0.005
	ProtD-Deca 007 / Alum	17	351	0.05
	ProtD-Deca 008 / Alum	15	48	0.32
14 Post 2	KLH-Deca / Alum	166	75222	0.002
	ProtD-Deca 007 / Alum	1260	24085	0.05
	ProtD-Deca 008 / Alum	2522	683	3.7
7 Post 3	KLH-Deca / Alum	495	63580	0.008
	ProtD-Deca 007 / Alum	3113	24889	0.13
	ProtD-Deca 008 / Alum	7313	1571	4.7

Conclusions

IgE peptide conjugated to Protein D was capable of inducing strong anti-peptide immune responses when adjuvanted with alum. The protein D conjugates induced

greater anti-peptide responses compared with those induced by the KLH conjugates, whilst inducing less anti-carrier responses that that induced by the KLH conjugates.

ProteinD-IgE peptide conjugates with a ratio of (8:1 peptide:carrier) performed better that those with a ratio of 2:1, in both the magnitude of the anti peptide IgG response and the relative proportions of peptide:carrier IgG responses. The conjugates with a ratio of 2:1 (peptide:carrier) nevertheless performed better than KLH in these respects.

Example 3, *Immunogenicity studies with Stanworth decapeptide conjugates formulated with oil in water adjuvants*

Three groups of 10 Balb/C mice were vaccinated with the human decapeptide conjugates produced according to the methods described in example 1 and 2. The vaccines comprised 25μg of antigen formulated with an oil in water emulsion adjuvant (produced as described in example 1.8) in 100μl volumes, and were given s.c on three occasions on days 0, 21, and 42 (V1, V2, and V3).

Table 5, *Vaccine formulations in example 3.*

Name of group	***Number of peptide:carrier***	***Adjuvant***
ProtD-Deca 007	2	O/W emulsion, 3D-MPL (2 μg), QS21 (5 μg)
ProtD-Deca 008	8	O/W emulsion, 3D-MPL (2 μg), QS21 (5 μg)
KLH-Deca	5	O/W emulsion, 3D-MPL (2 μg), QS21 (5 μg)

Bleedings were taken 21, 35, and 49 days post commencement of immunisation (21 post V1, 14 post V2, and 7 post V3).

The results for generation of antibody responses are shown in Table 6 for individual responses at time points 21 post V1, 14 post V2, and 7 post V3. Figure 3 (anti-decapeptide antibody responses and anti-carrier antibody responses) shows the comparison of the antibody responses induced by the three vaccines in pooled sera.

The isotype of the antibodies produced was investigated in the ELISA system using anti-mouse isotype monoclonal antibodies (see table 6). These studies were performed on pooled serum samples.

Table 6, Anti-decapeptide responses generated by antigen conjugates formulated with oil in water emulsion adjuvants (7 post V3)

	Anti-Decapeptide (μg/ml)				***Isotype %***		
	IgG1	*IgG2a*	*IgG2b*	*total*	*IgG1*	*IgG2a*	*IgG2b*
ProtD-Deca 007	223	52	22	296	75	17	7
ProtD-Deca 008	354	10	5	369	96	3	1
KLH-Deca	202	61	47	311	65	20	15

The ratio's of Mid-point titres of anti-peptide:anti-carrier IgG responses were measured (results see table 7). This ratio is a measure of the relative levels of anti-peptide or anti-carrier immune response which were generated by vaccination. A high ratio (>1) indicates that strong anti-peptide response is being generated with respect to that induced against the carrier.

Table 7, *Midpoint titers and ratio anti-deca versus anti-carrier response*

Time point	Vaccine formulation	Titre Mid point		Ratio
		IgG anti Deca	IgG anti carrier	IgG anti Deca : IgG anti carrier
21 Post 1	KLH-Deca / SBAS2	1811	239520	0.01
	ProtD-Deca 007 / SBAS2	1118	1343	0.8
	ProtD-Deca 008 / SBAS2	3641	1718	2.1
14 Post 2	KLH-Deca / SBAS2	24641	521684	0.05
	ProtD-Deca 007 / SBAS2	23319	27170	0.9
	ProtD-Deca 008 / SBAS2	36130	21601	1.7
7 Post 3	KLH-Deca / SBAS2	21260	336431	0.06
	ProtD-Deca 007 / SBAS2	26012	23615	1.1
	ProtD-Deca 008 / SBAS2	22759	11968	1.9

Conclusions

IgE peptide conjugated to Protein D was capable of inducing strong anti-peptide immune responses when adjuvanted with oil in water emulsions. The protein D conjugates induced greater anti-peptide responses compared with those induced by the

KLH conjugates, whilst inducing less anti-carrier responses that that induced by the KLH conjugates.

***Example 4**, Immunogenicity studies with dodecapeptide conjugates adsorbed onto alum*

The dodeca-peptide used in this example (PEP3) is the rodent equivalent of the human IgE peptide used previously (PEP2). This example, therefore, examines the use of ProteinD-IgE peptide conjugates in a self-antigen model. The conjugates were formulated as described in examples 1.4 and 1.5 above.

The vaccines comprised 25μg of antigen formulated with an alum adjuvant in 100μl volumes, and were given s.c. on three occasions on days 0, 21, and 42 (V1, V2, and V3).

Table 8, *Vaccine compositions*

Name of group	***Number of peptide:ProtD***	***Adjuvant***
ProtD-Deca 007	2	$Al(OH)_3$ (100 μg)
ProtD-Deca 008	8	$Al(OH)_3$ (100 μg)

Bleedings were taken 21, 35, and 49 days post commencement of immunisation (21 post V1, 14 post V2, and 7 post V3). The ratio's of Mid-point titres of anti-peptide:anti-carrier IgG responses were measured (results see table 9). This ratio is a measure of the relative levels of anti-peptide or anti-carrier immune response which were generated by vaccination. A high ratio (>1) indicates that strong anti-peptide response is being generated with respect to that induced against the carrier.

Table 9, *Midpoint titers and ratio anti-deca versus anti-carrier response*

Time Point	Vaccine formulation	Titre Mid point		Ratio
		IgG anti Dodeca	IgG anti carrier	IgG anti dodeca : IgG anti carrier
21 Post 1	ProtD - Dodeca 007	1	168	0.006
	ProtD - Dodeca 008	230	113	2.04
14 Post 2	ProtD - Dodeca 007	6	9970	0.001
	ProtD - Dodeca 008	1961	4791	0.41
7 Post 3	ProtD - Dodeca 007	205	21573	0.009
	ProtD - Dodeca 008	2980	8826	0.34

Conclusions

ProteinD-dodeca peptide conjugates adsorbed onto alum are capable of inducing anti-peptide immune responses in the context of a self-antigen model. In this respect the high ratio conjugate performed better than the low ratio construct.

Example 5, *Immunogenicity studies with dodecapeptide conjugates formulated with oil in water adjuvants*

The dodeca-peptide used in this example (PEP3) is the rodent equivalent of the human IgE peptide used previously (PEP2). This example, therefore, examines the use of ProteinD-IgE peptide conjugates in a self-antigen model. The conjugates were formulated as described in examples 1.4 and 1.5 above.

The vaccines comprised 25μg of antigen formulated with an oil in water emulsion adjuvant (produced according to example 1.8) in 100μl volumes, and were given s.c. on three occasions on days 0, 21, and 42 (V1, V2, and V3).

Table 10, *Vaccine compositions*

Name of group	*Number of peptide:ProtD*	*Adjuvant*
ProtD-Deca 007	2	O/W emulsion, 3D-MPL (2 μg), QS21 (5 μg)
ProtD-Deca 008	8	O/W emulsion, 3D-MPL (2 μg), QS21 (5 μg)

Bleedings were taken 21, 35, and 49 days post commencement of immunisation (21 post V1, 14 post V2, and 7 post V3). The ratio's of Mid-point titres of anti-peptide:anti-carrier IgG responses were measured (results see table 11). This ratio is a measure of the relative levels of anti-peptide or anti-carrier immune response which were generated by vaccination. A high ratio (>1) indicates that strong anti-peptide response is being generated with respect to that induced against the carrier.

Table 11, *Midpoint titers and ratio anti-dodeca versus anti-carrier response*

Time Point	Vaccine formulation	Titre Mid point		ratio
		IgG anti Dodeca	IgG anti carrier	IgG anti Dodeca : IgG anti protD
21 Post 1	ProtD - Dodeca 007 / SBAS2	219	828	0.26
	ProtD - Dodeca 008 / SBAS2	11195	3645	3.07
14 Post 2	ProtD - Dodeca 007 / SBAS2	6811	14628	0.47
	ProtD - Dodeca 008 / SBAS2	43173	20194	2.14
7 Post 3	ProtD - Dodeca 007 / SBAS2	13392	22595	0.59
	ProtD - Dodeca 008 / SBAS2	22008	17507	1.26

Conclusions

ProteinD-dodeca peptide conjugates adsorbed onto alum are capable of inducing anti-peptide immune responses in the context of a self-antigen model. In this respect the high ratio conjugate performed better than the low ratio construct.

Example 6, Immunogenicity studies with Stanworth decapeptide conjugates in mice

Groups of Balb/C mice were vaccinated in order to further investigate the benefits of the Protein D carrier over the use of KLH. Briefly, groups of 10 mice were vaccinated on three occasions with on days 0, 21 and 42 with vaccines conjugated, formulated and inoculated according to Table 1.

Bleeds were taken on days 35 and 56. Results for anti-peptide IgG responses are shown in Figure 4 and anti-carrier IgG responses are shown in Figure 5 .

The anti-peptide and anti-carrier immune responses were investigated using an ELISA described in section 1.8.

Table 12, *Vaccine formulations (all groups adjuvanted with alum (100μg))*

Group	*Antigen*	*Ratio peptide:carrier*	*Immunisation route*
1	PD/deca	8	subcut
2	PD/deca	8	intramuscular
3	PD/deca	4	subcut
4	PD/deca	4	intramuscular
5	KLH/deca	5	subcut
6	KLH/deca	5	intramuscular

Groups 1 -2 and 3-4 were produced as described in examples 1.5 and 1.4. Group 5-6 was produced as described in example 1.9

***Example** 7 Immunogenicity studies with decapeptide conjugates in monkeys*

Two groups of 5 Rhesus monkeys were vaccinated respectively with the PEP2 human decapeptide-protein D ,conjugates produced and formulated as described in example 1.4 and with PEP2 human decapeptide-KLH, conjugates produced and formulated as described in example 1.11.

The vaccine comprised 250μg of antigen in 1ml and were given on three occasions on day 0, 28 and 56.

Bleeds were taken on day 28, 42 and 70.

The anti-peptide responses were investigated using an ELISA described in section 1.8 (but the second reagent used is an anti-human IgG instead of an anti-mouse IgG).

Results for anti-peptide IgG response (days 28 and 42) are shown in Figure 6. A higher anticarrier response was also found for the KLH as compared to the Protein D conjugate.

Conclusions.

IgE peptide conjugated to Protein D induced a significantly greater anti-peptide response compared to the titer induced by the KLH conjugate.

Example 8

In a subsequent series of experiments Protein D was evaluated as a carrier for successive boost using KLH as a positive control.

Material

Four groups of 10 female BALB/c mice were primed and boosted as follows (LAS 98571):

1. 25 µg KLH-decapeptide (EKLH00B) intramuscular (IM)
2. 25 µg KLH-decapeptide subcutaneous (SC)
3. 25 µg Protein D-decapeptide (PDGCH010C) intramuscular (IM)
4. 25 µg Protein D-decapeptide subcutaneous (SC)

Six immunisations were performed at day 0, 21, 42, 150, 177 and 205 and constructs were formulated on Al(OH)3 100 µg.

Bleedings were made at day 14 after injection and on day 150 before injection.

The anti-carrier and anti-peptide antibody responses were measured in ELISA. There is a striking difference between the antibody responses made from KLH-deca and Protein D-deca (PD-deca) constructs (figure 7).

KLH-deca induced a very strong anti-carrier response, whereas the anti-decapeptide response was quite low. The opposite can be said for PD-deca.

The evolution of the titres was similar be it KLH-deca or PD-deca immunisation.

After the boost of the response at 14 post III, a 60% drop of antibody levels at day 150 (i.e. 108 days post III).

A fourth injection again increased the antibody titres, although often not to the same level as at day 14 post III.

In the case of PD-deca, a fifth injection one month later was necessary to reach post III titres.

When comparing SC and IM route for immunisation it appears that the IM injection gave rise to higher antibody titres than SC.

Subsequent injections may lead to an undesired induction of IgE antibodies, especially if the individual is predisposed as in the case of allergy. The induction of anti-carrier IgE was therefore measured (Figure 8).

As for total Ig, the IgE anti-KLH levels were much higher than IgE anti-PD.

Conclusion

Protein D allows a higher anti-decapeptide antibody response than KLH, while keeping a moderate level of anti-carrier antibodies, both total Ig and IgE.

It is of note that although the constructs have approximately the same molar ratio of decapeptide/carrier (about 4-5 decapeptides/carrier), the sizes of the carriers are very different. KLH has a molecular weight of +/- 390 000 against the 40 000 for PD. Therefore, 25 μg of construct (the injected dose) will contain around 0.5 μg or 2 μg of decapeptide if it is KLH-deca or PD-deca respectively.

For this reason, PD can be said to be a "better" carrier than KLH, since more peptide can be administered with the same amount of construct.

The IM route for injection appears preferable. It induces higher Ig levels and somewhat lower levels of IgE.

Example 9: *Protein D – P15 (Allergy peptide)*

A mimotope of P1 was synthesised CLEDGQVMDVDLL (P15) (SEQ ID No. 10) which was conjugated to Protein D using techniques described above in Example 1.

ELISA methods

Anti-peptide or Anti-peptide carrier ELISA

The anti-peptide and anti-carrier immune responses were investigated using an ELISA technique outlined below. Microtiterplates (Nunc) are coated with the specific antigen in PBS (4° overnight) with either: Streptavidin at 2μg/ml (followed by incubation with biotinylated peptide (1μM) for 1 hour at 37°C), Wash 3X PBS-Tween 20 0.1%. Saturate plates with PBS-BSA 1%-Tween 20 0.1% (Sat buffer) for 1 hr at 37°. Add 1° antibody = sera in two-step dilution (in Sat buffer), incubate 1 hr 30 minutes at 37°. Wash 3X. Add 2° anti-mouse Ig (or anti-mouse isotype specific monoclonal antibody)

coupled to HRP. Incubate 1 hr at 37°. Wash 5X. Reveal with TMB (BioRad) for 10 minutes at room temperature in the dark. Block reaction with 0.4N H_2SO_4.

Method for the Detection of Anti-Human IgE Reactivity in Mouse Serum (IgE plate bound ELISA)

ELISA plates are coated with human chimaeric IgE at 1μg/ml in pH 9.6 carbonate/bicarbonate coating buffer for 1 hour at 37°C or overnight at 4°C. Non-specific binding sites are blocked with PBS/0.05% Tween-20 containing 5% w/v Marvel milk powder for 1 hour at 37°C. Serial dilutions of mouse serum in PBS/0.05% Tween-20/1% w/v BSA/4% New Born Calf serum are then added for 1 hour at 37°C. Polyclonal serum binding is detected with goat anti-mouse IgG-Biotin (1/2000) followed by Streptavidin-HRP (1/1000). Conjugated antibody is detected with TMB substrate at 450nm. A standard curve of PTmAb0011 (anti IgE antibody) is included on each plate so that the anti-IgE reactivity in serum samples can be calculated in μg/ml.

Human Basophil Assays

Two types of assay were performed with human basophils (HBA), one to determine the anaphylatogenicity of the monoclonal antibodies, consisting of adding the antbodies to isolated PBMC; and a second to measure the inhibition of *Lol P I* (a strong allergen) triggered histamine release be pre-incubation of the HBA with the monoclonal antibodies.

Blood is collected by venepuncture from allergic donors into tubes containing 0.1 volumes 2.7% EDTA, pH 7.0. It is then diluted 1/2 with an equal volume of HBH medium containing 0.1% human serum albumin (HBH/HSA). The resulting cell suspension is layered over 50% volume Ficoll-Paque and centrifuged at 400g for 30 minutes at room temperature. The peripheral blood mononuclear cell (PBMC) layer at the interface is collected and the pellet is discarded. The cells are washed once in HBH/HSA, counted, and re-suspended in HBH/HSA at a cell density of 2.0×10^6 per ml. 100μl cell suspension are added to wells of a V-bottom 96-well plate containing 100μl diluted test sample or monoclonal antibody. Each test sample is tested at a

range of dilutions with 6 wells for each dilution. Well contents are mixed briefly using a plate shaker, before incubation at 37°C for 30 minutes.

For each serum dilution 3 wells are triggered by addition of 10µl Lol p I extract (final dilution 1/10000) and 3 wells have 10µl HBH/HSA added for assessment of anaphylactogenicity. Well contents are again mixed briefly using a plate shaker, before incubation at 37°C for a further 30 minutes. Incubations are terminated by centrifugation at 500g for 5 min. Supernatants are removed for histamine assay using the standard histamine. Control wells containing cells without test sample are routinely included to determine spontaneous and triggered release. Wells containing cells were lysed by two cycles of freeze/thawing to determine total cell histamine.

The results are expressed as following:

Anaphylactogenesis assay

Histamine release due to test samples =

% histamine release from test sample treated cells – % spontaneous histamine release.

Blocking assay

The degree of inhibition of histamine release can be calculated using the formula:

% inhibition

= 1 -(histamine release from test sample treated cells*) x 100

(histamine release from antigen stimulated cells*)

Values corrected for spontaneous release.

Example 10, *Immunisation of mice with P15 conjugates (P15 -PD) induces production of anti-human IgE antibodies.*

The Protein D conjugate comprising the mimotope P15 (25µg protein/dose), were administered into groups of 10 BalbC mice, adjuvanted with and oil in water emulsion containing QS21 and 3D-MPL described in WO 95/17210 . Boosting was performed on day 21 and on day 42 and sera can be harvested on day 42 and 56.

The immune response anti-peptide and anti-plate bound IgE was followed using methods described above.

Results

The results for anti peptide and anti-IgE responses measured at day 14 post third vaccination are shown in the table below.

P15 Immunogenicity results

Mimotope conjugate	Anti-peptide responses (Mid point titre)			Anti-IgE responses (μg/ml (PTmAb0011))		
	Average	Std Dev.	Geomean	Average	Std Dev.	Geomean
P15-PD (n=16)	41391	26858	36154	1.6	4.5	0.3

Example 11, *Anti-IgE induced in mice after immunisation with conjugate are non anaphylactogenic*

Several dilutions of complete sera or IgG purified from conjugate immunised mice can be tested in presence of basophils from freshly harvested peripheral blood from allergic patients.

The anaphylactogenicity can be evaluated by the measuring of the histamine released induced by the antibodies to be tested as described below:

- Erythrocytes are removed from peripheral blood on glucose dextran gradient
- Cells are washed and plated with samples to be tested (for example allergen, antibodies, allergen plus antibodies,...)
- After incubation , supernatants are collected and histamine release is measured according to manufacturer's instructions (Immunotech , histamine enzyme immunoassay kit)

Antiserum generated with P15-PD was shown not to be anaphylactogenic.

Example 12, *Anti-IgE induced in mice after immunisation with conjugate are capable of blocking IgE mediated histamine release induced by allergen triggering of basophil from an allergic patient.*

Histamine release can be measured in basophil samples triggered with various concentrations of allergen in presence or absence of several dilutions of complete sera or IgG purified from conjugate immunised mice. Blocking activity of anti-P15 antibodies in the antiserum was evaluated by the measuring of the inhibition of the histamine release induced by the allergen. Histamine release and inhibition was measured as described in example 3. As P15 is a mimotope of P1, PTmAb0011 was used as a control as it is known to bind to the same epitope (P1). The results are shown in the table below.

Histamine release inhibition from allergic human basophils

Antiserum	Dilution	% inhibition of histamine release
P15-PD (mouse 4.12)	1/30	79
P15-PD (mouse 4.5)	1/30	57
PTmAb0011	0.1μg/ml	56
PTmAb0011	1μg/ml	90
anti-PD serum	1/30	40

Example 13: GnRH – Protein D

In a manner analagons to Example 1 GnRH peptide EHWSYGLRPG (SEQ ID NO. 1) was synthesised with an N terminal linker CPPPPSS (SEQ ID NO. 18) and conjugated to protein D via a succinimide – maleimide cross-linker. Four formulations were evaluated to assess anti GnRH antibodies and Testerone levels in male rabbits.

Intra muscular injections of vaccine (5 male rabbits (4 months)/group) were given at 0, 1 and 3 weeks and bleedings performed 7 days post I, 7 days post II, 7 days post III and 14 days after the third injection and the anti GnRH antibody levels determined by an ELISA assay.

Formulations tested

1. PD-GnRH (10μg) + oil/water emulsion ISA703 (Montenide s.a.)
2. PD-GnRH (100μg) + oil/water emulsion ISA703 (Montenide s.a.)
3. PD-GnRH (10μg) QS21 cholesterol liposomal formulation with 3D-MPL (150μ) internalised within the liposome (see WO98/15287) adsorbed on aluminium hydroxide (500μg).
4. PD-GnRH (100μg) formulated as formulation 3.

The average antibody titres are shown in the table below.

FORMULATIONS		PRE	7d post I	7d post II	14d post II	7d post III	14d post III
PD-GnRH (10)ISA703	PD ISA703	935	815	734	6301	36771	75620
PD-GnRH (100)ISA703	PD ISA703	836	750	733	18106	53345	80272
PD-GnRH(10)MPL in (150)/DQS21 (150) Alum (500)	PD AS5i	859	889	3328	9189	44823	52024
PD-GnRH(100)MPL in (150)/DQS21 (150) Alum (500)	PDAS5i	849	514	4289	11577	80098	71305

Testerone levels 14 days post III injection were no longer measurable.

***Example 14**: Conjugates of LB1 – Protein D*

Non typeable Haemophilus influenzae are encapsulated gram-negative bacteria which are common human upper respiratory tracts inhabitants frequently associate with otitis media, bronchitis and other infections of mucosal surfaces.

Colonisation of the respiratory tract mucosa is the first and next step the presumed pathogenesis of NTHi infection.

NTHi strains contain bacterial fimbriae defined as non flagellar proteinaceous surface appendages.

The gene coding for the fimbrial subunit protein-fimbrin- in non typeable H.influenzae 1128 was isolated, cloned and sequenced by L. Bakaletz. One constitutive peptide of the fimbrin: LB1 was identified. The amino acid sequence shows one important constitutive peptide (19 aa): LB1 with presumed B-cell determinants. LB1 is derived from the N-terminal half of the mature P5-fimbrin protein. Three major groups, based on amino acid sequence diversity, were identified. Approximately 75% of NTHi strains belong to group 1. A vaccine including two NTHi antigens (LB1 and PD) known to play a role in pathogenesis of otitis media could be interesting. A possible approach was to synthesize LB1 conjugates using protein D as carrier.

The coupling reagent is a selective heterobifunctional crosslinker, one end of the compound activating amino group of the protein carrier by a succinimidyl ester and the other end coupling sulfhydryl group of the peptide by a maleimido group. The reactional scheme is described previously.

The maleimide group is most selective for sulfhydryl groups when the pH of the reaction mixture is kept between 6.5 and 7.5. At pH 7, the rate of reaction of maleimides with sulfhydryl is 1000-fold faster than with amines. A stable thioether linkage between the maleimide group and the reacted sulfhydryl is formed which cannot be cleaved under physiological conditions.

LB1gr1 peptide was synthesized by Eurogentec. The following amino acid sequence was coupled though an additional C-terminal cysteine via maleimide to protein D (PD).

Sequence	group	Strain
Acetyl-RSDYKFYEDANGTRDHKKGC-NH2 (SEQ ID NO. 19)	LB1gr1	NTHi 1128

PD-LB1 conjugates were prepared as follows:

10 mg of PD (0.25 μmole) was diluted to 2.5 mg/ml with 100 mM phosphate buffer pH7.2. A large excess of heterobifunctionnal cross-linking reagent GMBS (N-[μ-

maleimidobutyryloxy]succinimide ester) (equivalent to activate 2 x 36 lysine) was dissolved in 150 µl of DMSO. The resulting GMBS solution was added to the PD solution with stirring and allowed to react for 60 minutes at room temperature. In order to remove excess reagents and GMBS by-products, the activated PD was purified by gel filtration (Toyopearl HW-40, XK 16/40, elution with 100 mM phosphate buffer pH 6.8). LB1 peptide (2.5 mg/ml) was dissolved in acetic acid 0.1M and added to the activated protein solution. The conjugation reaction is allowed to continue for one hour at 25°C and followed by a quenching step with cysteine during 30 minutes at the same temperature. During the conjugation step, a slight precipitation was observed. After filtration, in order to remove excess peptide, the conjugate solution was dialysed against NaCl 150 mM or 100 mM phosphate buffer pH 6.8 with or without Tween 80 0.1%. The PD-LB1 conjugate solution is then filtered through a sterile 0.22 µm membrane.

Several conjugation assays using the maleimide chemistry were realised to study the influence of the PD / LB1 ratio and of Tween 80 on the solubility of the conjugate. The number of activated sites before and after conjugation was measured by the Ellman's reaction. Addition of 7 to 8 LB1 peptides on PD gives soluble conjugates with a good yield and without need of Tween 80 during the coupling step (Table below). Dialysis against NaCl seems to increase insolubility of the conjugate.

Batch	Initial ratio PD / peptide	Tween 80 (0.1 %)	CONJUGATE	Yield	Dialysis
PD-G-LB1-02	1 / 4	-	Soluble	57%	NaCl 150 mM
PD-G-LB1-03	1 / 6	-	Soluble	60.8%	NaCl 150 mM
PD-G-LB1-05	1 / 7	-	Soluble	**84.7%**	Phosphate buffer 100mM,pH 6.8
PD-G-LB1-06	1 / 7	+	Soluble	64.6%	Phosphate buffer 100 mM, pH 6.8
PD-G-LB1-04	1 / 8	-	+/- soluble	12.9%	NaCl 150 mM
PD-G-LB1-07	1 / 8	+	Soluble	**71.8%**	Phosphate buffer 100 mM, pH 6.8
PD-G-LB1-11	1 / 8	+	Soluble	**74%**	Phosphate buffer 100 mM, pH 6.8
PD-G-LB1-13	1 / 8	-	Soluble	**80.8%**	Phosphate buffer 100 mM, pH 6.8
PD-G-LB1-08	1 / 9	+	Soluble	36.5%	Phosphate buffer 100 mM, pH 6.8
PD-G-LB1-12	1 / 9	+	+/- soluble	15.4%	Phosphate buffer 100 mM, pH 6.8
PD-G-LB1-09	1 / 10	+	Soluble	45.4%	Phosphate buffer 100 mM, pH 6.8
PD-G-LB1-10	1 / 12	+	+/- soluble	16.7%	Phosphate buffer 100 mM, pH 6.8
PD-G-LB1-01	1 / 12	-	insoluble	/	NaCl 150 mM

Table 1: PD-G-LB1 conjugates

Presence of LB1 peptide on the carrier was confirmed by SDS-PAGE followed by coomassie blue staining or transfer on nitrocellulose followed by immunodectection with anti-LB1 antibodies.

Claims

1. An immunogen comprising a peptide and Protein D from Haemophilus Influenzae, or a fragment thereof as a carrier.
2. An immunogen as claimed in claim 1, wherein the peptide is between 2-50 amino acid residues in length.
3. An immunogen as claimed in claim 1 or 2, wherein said peptide is derived from one of an IgE epitope or mimotope, Gonadotrophin hormone releasing hormone or mimotope thereof, a fragment of the amyloid precursor protein or mimotope thereof.
4. An immunogen as claimed in any of claims 1to 3 wherein the ratio of peptide: protein D carrier is 1:20.
5. An immunogen as claimed in claim 4 wherein the ratio of peptide:protein D carrier is 2:10 peptides per protein D carrier.
6. An immunogen as claimed in any of claims 1 to 5, wherein the peptide is Aβ43 from the amyloid precursor protein or a fragment thereof.
7. An immunogen as claimed in claim 6 wherein the fragments are peptides selected from the group of peptides incorporating residues Aβ1-5, 1-12, 13-28, 17-28 and 33-42.
8. An immunogen as claimed in any one of claims 1 to 5 comprising the sequence EHWSYGLRPG as a tandem repeat conjugated to protein D through a central cysteine.
9. An immunogen as claimed in any of claims 1 to 5 derived from an IgE epitope selected from the group of peptides having the following sequences:

   KTKGSGFFVF

   EDGQVMDVD

   STTQEGEL

   SQKHWLSDRT

   GHTFEDSTKKCADSNPRGV
10. An immunogen as claimed in any of claims 1 to 5 wherein the mimotopes are selected from the group having the following sequences:

    CADSNPRGV

CLEDGQVMDVDLL-NH2

CSTTQEGELA- NH2

CSQKHWLSDRT- NH2

11. An immunogen as claimed herein wherein the protein D carrier is conjugated to a plurality of discrete peptides.

12. A vaccine comprising an immunogen as claimed herein and a pharmaceutically acceptable excipient.

13. A vaccine as claimed in claim 12 additionally comprising an adjuvant.

14. A vaccine as claimed in claim 13 wherein the adjuvant is selected from Saponin adjuvants, lipid A or derivative thereof, aluminium salt, oil in water emulsions, liposomes or combinations thereof.

15. A vaccine as claimed in any of claims 12 to 14 for use in medicine.

16. An immunogen as claimed in any of claims 1 to 11 for use in medicine.

17. Use of an immunogen as claimed herein, in the manufacture of a medicament, for the treatment or prophylaxis of an infectious or chronic disease.

18. A method of manufacturing an immunogen as claimed in any of claims 1 to 11 comprising the step of conjugating a peptide to protein D or a fragment thereof.

19. A method of manufacturing a vaccine as claimed in any of claims 12 to 15 comprising formulating an immunogen any of claims 1 to 11 with a pharmaceutically acceptable excipient.

20. A method of treating a patient suffering from or susceptible to a chronic or infectious disease comprising administering a safe and effective amount of vaccine or immunogen as claimed herein.

Figure 1 of 8, *Solubility of Protein D/peptide conjugates of different ratios*

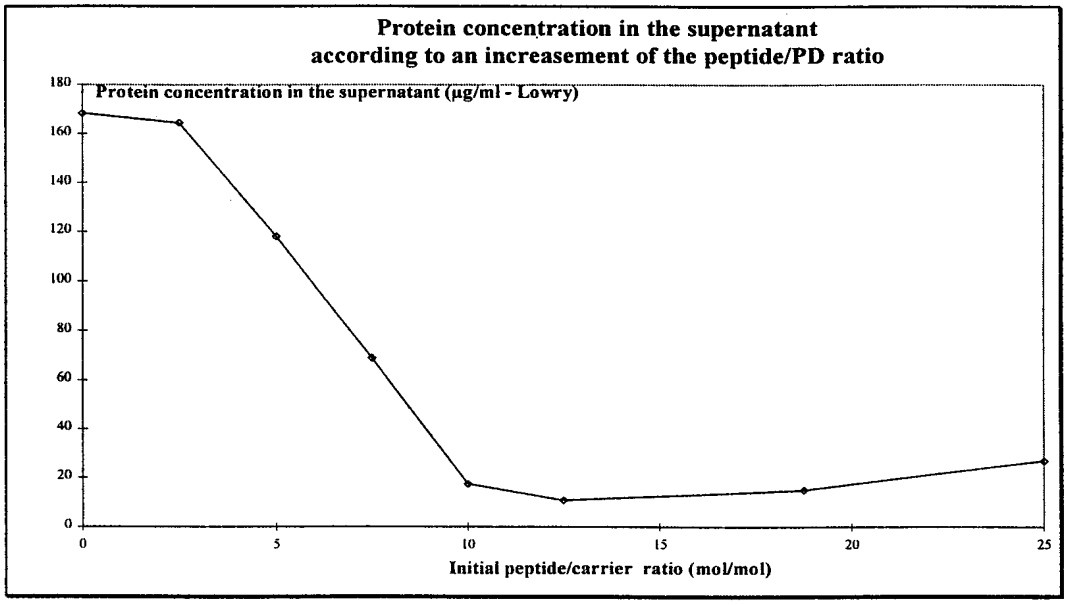
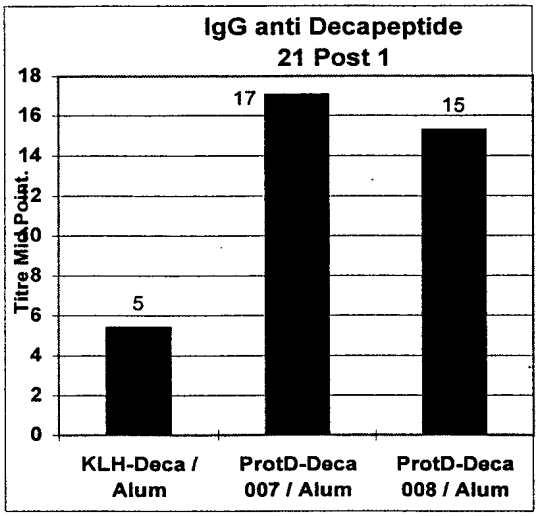
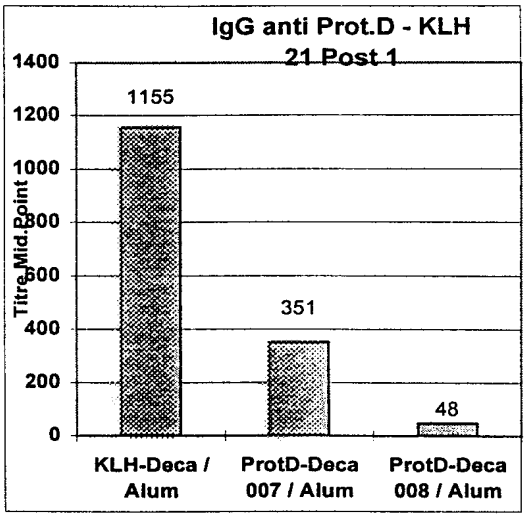
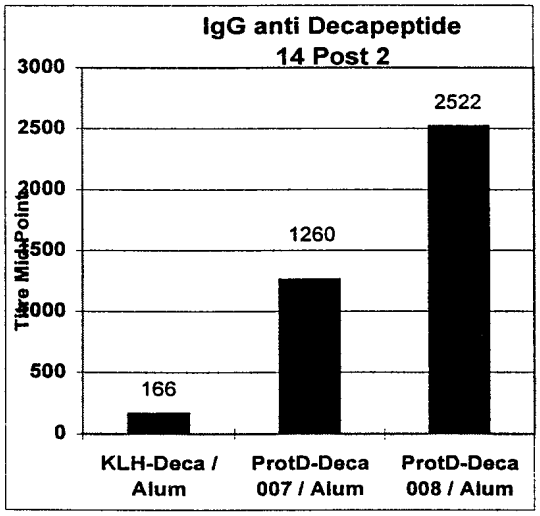
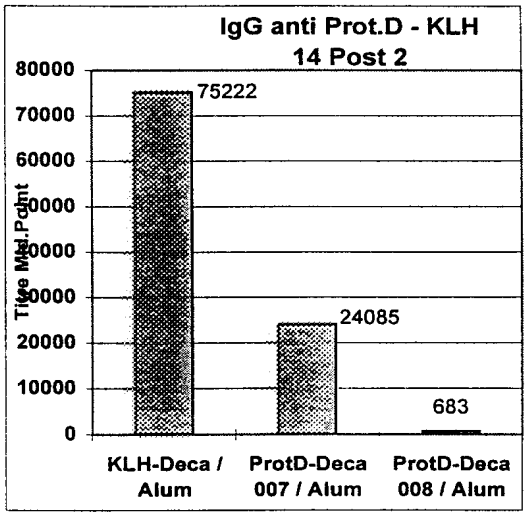


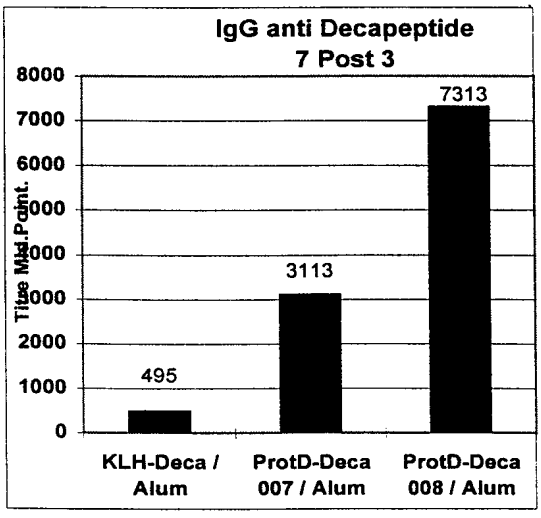
Figure 2 of 8, Total anti-decapeptide and anti-carrier IgG (Mid point titre)











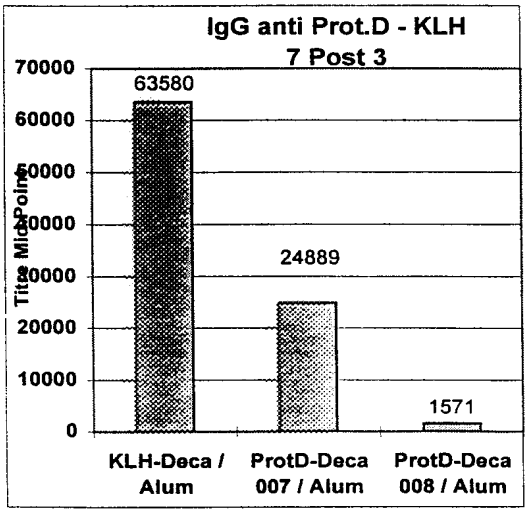


Figure 3 of 8, Total anti-decapeptide and anti-carrier Ig (Mid point titre) with vaccines comprising oil in water emulsion adjuvants.

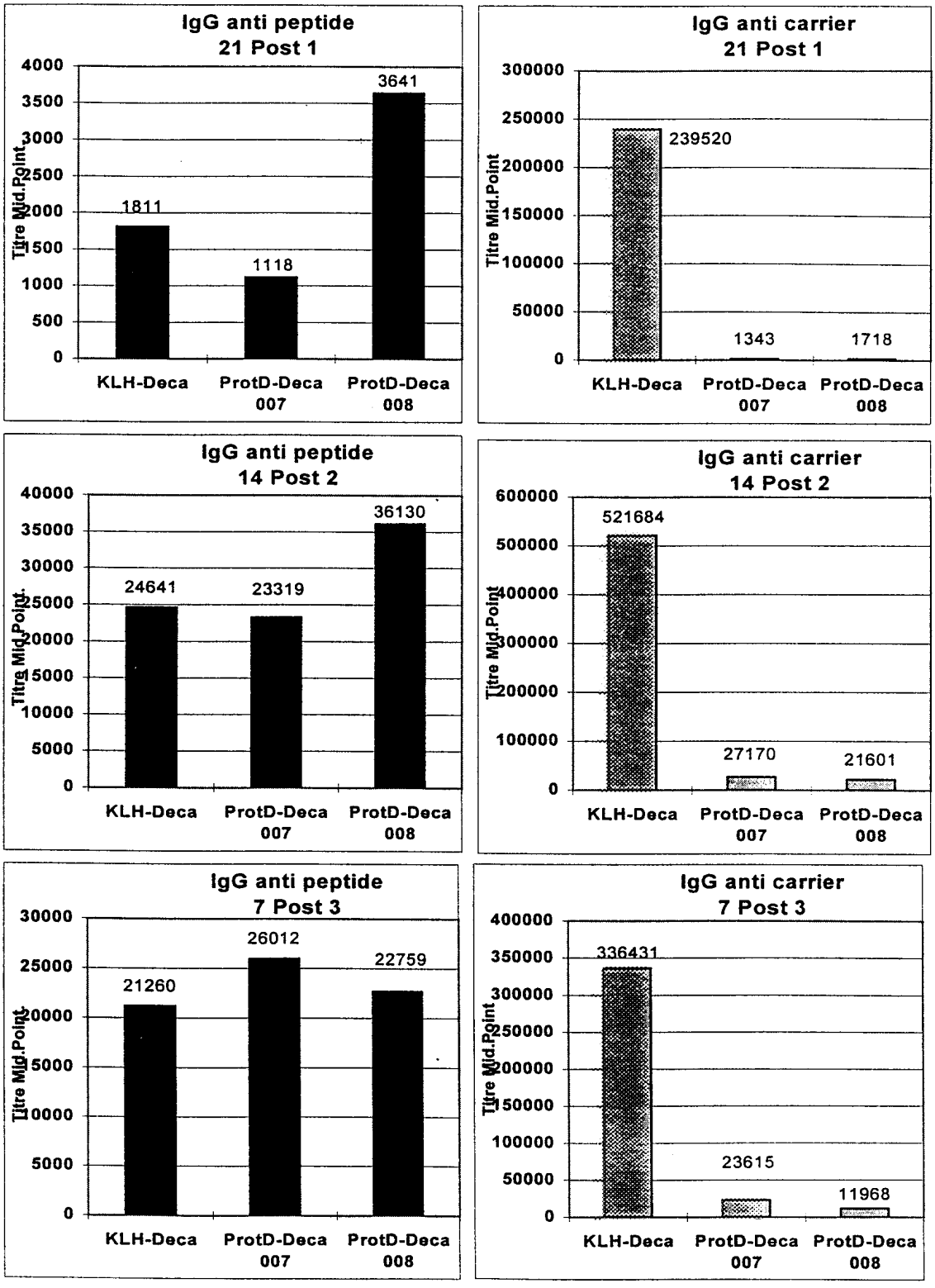


Figure 4 of 8, Anti-decapeptide IgG response

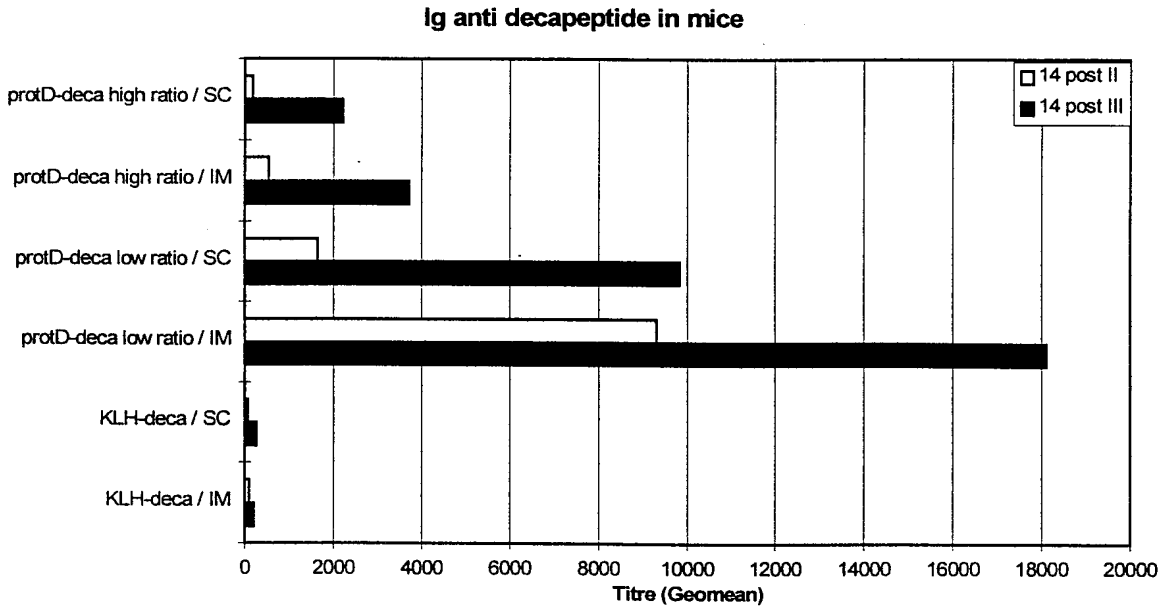


Figure 5 of 8, Anti-Carrier IgG response

5

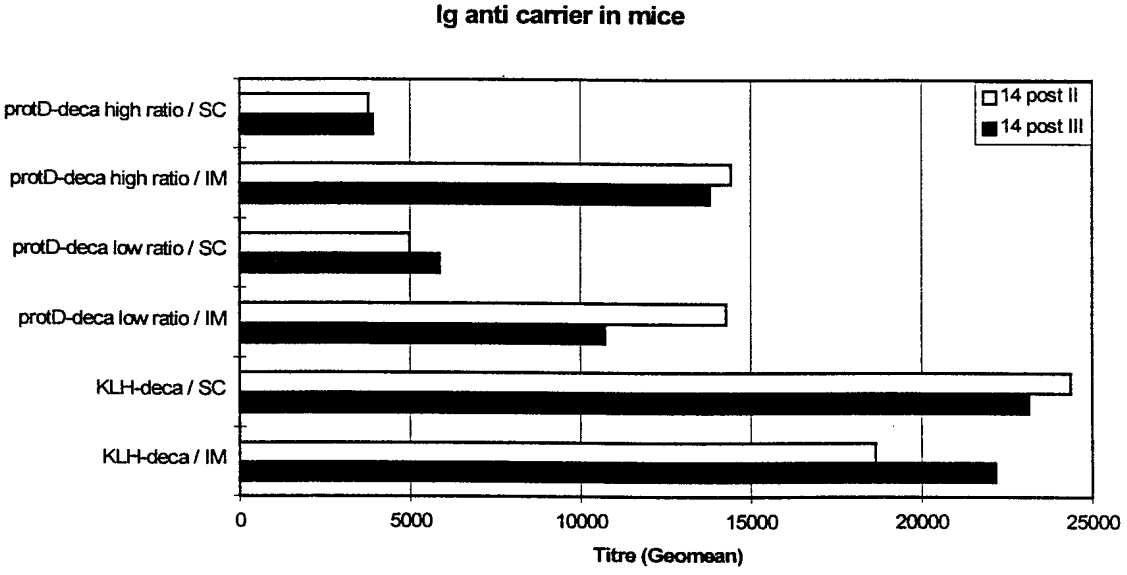


Figure 6 of 8, Anti-decapeptide IgG response.

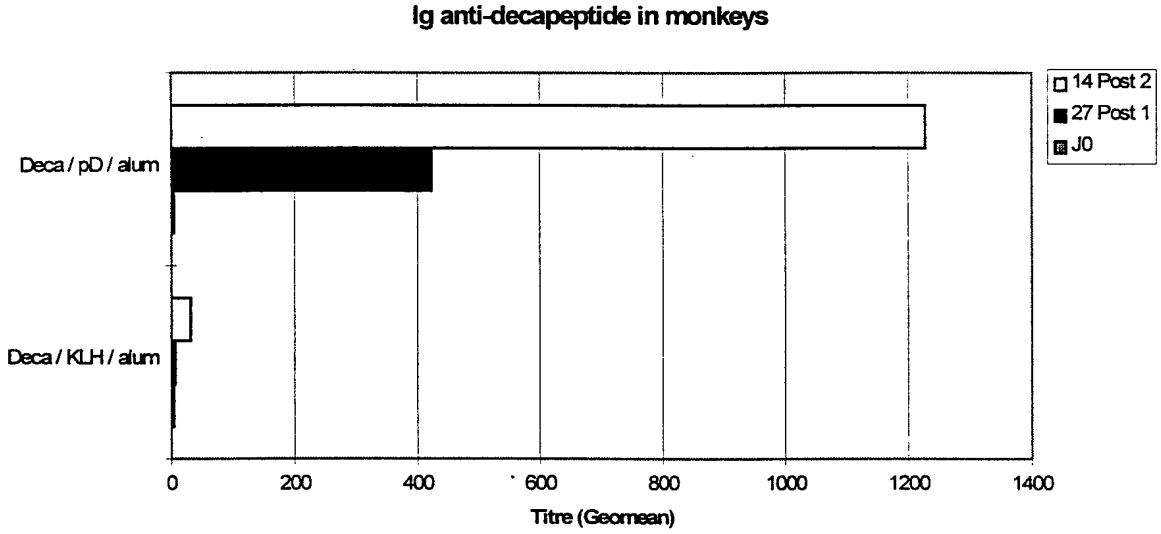
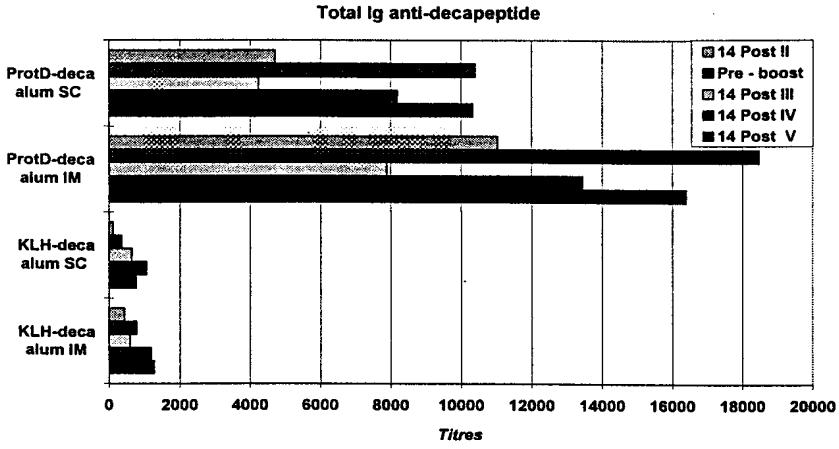


Figure 7 of 8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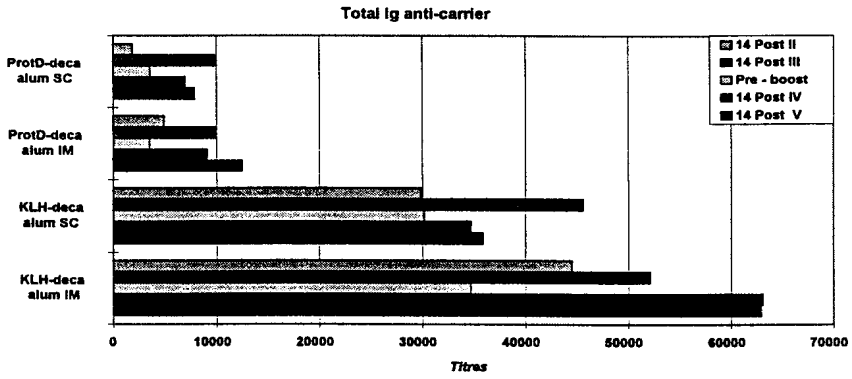
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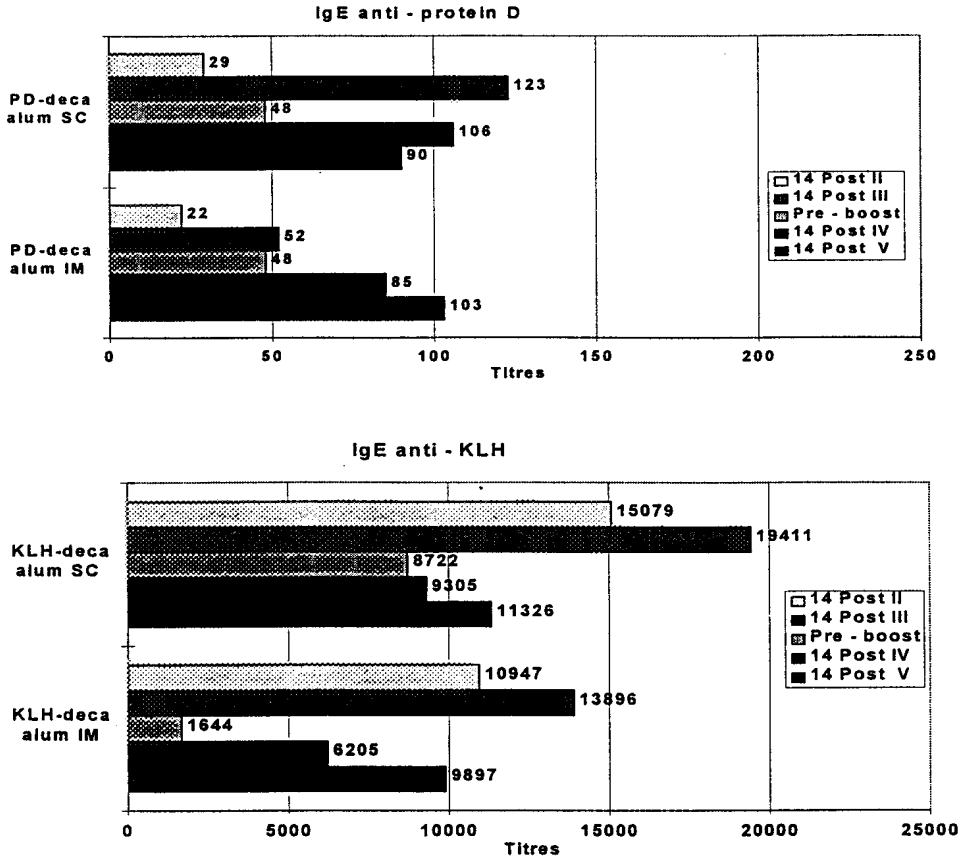
Figure 8 of 8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INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/EP 00/01457

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/385 A61K39/00 A61P31/00 //C07K16/00,C07K14/285, C07K14/575

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data, MEDLINE, CANCERLIT, AIDSLINE, LIFESCIENCES, EMBASE, SCISEARCH, BIOSIS, WPI Data, EPO-Internal, PAJ, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 858 677 A (FORSGREN ARNE) 12 January 1999 (1999-01-12) column 3, line 27-49 ---	1,12-20
A	AKKOYUNLU M ET AL: "The acylated form of protein D of Haemophilus influenzae is more immunogenic than the nonacylated form and elicits an adjuvant effect when it is used as a carrier conjugated to polyribosyl ribitol phosphate." INFECTION AND IMMUNITY, (1997 DEC) 65 (12) 5010-6. , XP002142903 page 5010, right-hand column, paragraph 3 -page 5011, left-hand column, paragraph 1 page 5011, left-hand column, paragraphs 5,7 page 5015, left-hand column, paragraph 2 --- -/--	1-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 July 2000	31/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Covone, M

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INTERNATIONAL SEARCH REPORT

Internatl Application No
PCT/EP 00/01457

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	WO 97 35613 A (SVENSON STEFAN) 2 October 1997 (1997-10-02) page 2, line 10 -page 4, line 15 claims 2,11-14	1-20
A	EP 0 293 530 A (PROTEUS MOLECULAR DESIGN) 7 December 1988 (1988-12-07) claims 1,6,7,12	8,12-19
A	WO 97 31948 A (CIBA GEIGY AG ;KRICEK FRANZ (AT); STADLER BEDA (CH)) 4 September 1997 (1997-09-04) page 4, line 6-25 example 8 figures 3,4	9,12-20
P,X	WO 99 16884 A (GODART STEPHANE ANDRE GEORGES ;SMITHKLINE BEECHAM BIOLOG (BE); BRU) 8 April 1999 (1999-04-08) examples 1.2,5 claims 1,7,16-20	1,2, 12-20

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Information on patent family members

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